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Serum proteome of dogs at subclinical and clinical onset of canine leishmaniosis

Running title: **Serum proteome in canine leishmaniosis**

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Abstract

The objective of this study was to identify changes in serum proteome in dogs that may occur after an experimental infection at subclinical and clinical stages of canine leishmaniosis (CanL).

For this purpose, canine pre- and post-infection with *Leishmania infantum* serum proteomes in the same dogs were analysed by a high-throughput label-based quantitative LC-MS/MS proteomic approach. A total of 169 proteins were identified, and 74 of them including complement C8 alpha chain, adiponectin, transferrin sphingomyelin phosphodiesterase acid like 3A, and immunoglobulins showed different modulation between the different stages of CanL. These proteins could be considered as potential biomarkers in serum of early diagnostic or disease progression in CanL. Additionally, biological pathways modulated during CanL such as blood coagulation or gonadotropin-releasing hormone receptor were revealed, which could help to understand the pathological mechanisms of the disease.

Keywords: biomarkers, dog diseases, Leishmania, proteomics, SWATH-MS.

1. Introduction

Visceral leishmaniosis is a global zoonosis caused by *Leishmania spp* parasites that is considered a major threat to human and animal health (Kuleš et al., 2016). Dogs are the main reservoirs of the disease (Miró et al., 2017), and canine leishmaniosis (CanL) is endemic in more than 70 countries (Laia Solano-Gallego et al., 2011). CanL can produce a wide range of clinical presentations since it can potentially affect any organ or tissue, ranging from subclinical to fatal illness. Thus, the development of CanL is complex and often unpredictable, being their pathological mechanisms not fully understood.

The use of proteomics could bring more light and comprehension in the disease mechanisms, since it allows us to compare a high number of proteins simultaneously. This information can be used in order to identify the pathways and biological processes affected along the course of the disease. Proteomics has been widely used in the last few years in several canine diseases such as babesiosis (Kuleš et al., 2014), glomerular disease (Nabity et al., 2011), and leishmaniosis (Escribano et al., 2016; Martínez-Subiela et al., 2017), among others. The proteomics approach has also been employed for the identification of disease specific serum biomarkers and candidate vaccine protective antigens (Artigas-Jerónimo, De La Fuente, & Villar, 2018; Contreras et al., 2018).

The clinical and clinicopathological findings observed in CanL are the consequence of complex interactions between the parasite and the genetic and immunological response of the dog, making CanL a very pleomorphic disease (L. Solano-Gallego et al., 2009). Dogs present clinical CanL when they exhibit clinical signs and/or clinicopathological abnormalities and a confirmed *L. infantum* infection. There are established guidelines such as the LeishVet guidelines (Solano-Gallego et al., 2011) published, in which animals are classified in different clinical stages according to their physical examination and laboratory findings. For example, in this guide, the 'Moderate disease, Stage IIa' includes dogs with medium to high positive antibody titers and clinicopathological abnormalities that may include hypoalbuminemia, hyperglobulinemia, mild non-regenerative anemia, and a normal renal profile (creatinine < 1.4 mg/dl and non-proteinuric profile with UPC < 0.5). Externally, dogs in clinical stage II may present anorexia, weight loss, peripheral lymphadenomegaly, cutaneous lesions, fever, or epistaxis, among other clinical signs.

On the other hand, there are dogs with confirmed *L. infantum* infection that do not present clinical signs or clinicopathological abnormalities, which are considered subclinical infected or healthy infected asymptomatic dogs. Before the appearance of clinical signs of CanL in dogs, a subclinical phase usually occurs that can last a few months which can evolve into a disease with clinical manifestations. In previous reports (Martinez-Subiela, Strauss-Ayali, Cerón, & Baneth, 2011; Paltrinieri, Gradoni, Roura, Zatelli, & Zini, n.d.), a moderate increase of acute phase proteins such as C-reactive protein and ferritin has been observed previous to the appearance of clinical signs. Diagnosis of these dogs in a subclinical stage is of high importance as they are

reservoirs for other dogs, humans and other species (Ashford et al., 1993). Additionally, the early detection and close follow up of these dogs would allow them an early treatment that may result in a better quality of life and expectancy.

The objective of this study was to analyse the serum proteome changes that occur during the subclinical and clinical stages of CanL in the same dogs, using an experimental infection model. This study could help us in the understanding of the CanL physiopathology and the changes that are responsible of the onset of the disease and could potentially help to identify new early biomarkers of the disease progression and pathology.

2. Material and Methods

2.1. Animals, sample procedures and experimental design

A total of 4 beagle dogs bought from Isoquimen S.L. (Barcelona, Spain) were involved in this study, which was made in the University of Murcia installations (south-eastern Spain). All dogs were negative to the presence of *L. infantum* (tested by Enzyme-Linked ImmunoSorbent Assay (ELISA) and real-time polymerase chain reaction (qPCR) of lymph node and bone marrow aspirates) and *Dirofilaria immitis*, *Anaplasma Phagocytophilum*, *Borrelia Burgdorferi*, and *Ehrlichia Canis* antibodies (using the IDEXX SNAP® 4Dx® Test, IDEXX laboratories, IDEXX Europe B.V., Hoofddorp, The Netherlands).

All the procedures were approved by University of Murcia Animal Ethics Committee (protocol number: 276/2016) and the Regional government of Murcia (identification code number: A13151002), and were performed in compliance with the law RD53/2013 about animal experimentation in Spain.

Dogs were submitted to an experimental infection with *L. infantum* through intravenous injection with 1×10^6 stationary-phase infective *L. infantum* (MCAN/BR/00/BA262) promastigotes. *L. infantum* parasites (amastigotes form) were isolated from spleen of an experimentally infected hamster and cultured in Schneider's *Drosophila* medium (Biowest) supplemented with 10% of inactivated fetal bovine serum (iFBS) and 50 µg/mL gentamicin at 26°C. Promastigotes derived

from the splenic amastigotes were cultured in modified RPMI-1640 medium supplemented with 20% of iFBS, and 50 µg/mL gentamicin at 26°C. Parasite shape and motility was examined by microscopic examination. With the aim of avoiding re-infections by *L. infantum* during the study, all dogs were externally dewormed with an anti-sandfly activity insecticide (permethrin 500mg/ml and imidacloprid 100mg/ml, Advantix pipettes, Bayer®) every three weeks and internally dewormed (50mg praziquantel, 50 mg pyrantel and 150 mg febantel; Prazitel tablets, Ecuphar®) in accordance with manufacturer's instructions. In addition, dogs were fed with an equilibrated and high quality dry dog food (Affinity Libra Adult with Chicken, Affinity®) throughout the study.

Blood samples (10 ml) were collected by venipuncture of the jugular vein in tubes containing a coagulation activator and a gel separator and kept at room temperature (25ºC) until visible clot reaction. Samples were centrifuged at 3.500 rpm for 5 min and serum were separated and stored at -80ºC until analysis. During the study, external clinical examination –performed by a licensed veterinarian (LB)-, haematology, and serum biochemistry analyses were performed monthly. Quantitative PCR was performed in bone marrow at least every two months..

For the present study, blood samples from three different time points (pre-infection, T0, and 2 different times post-infection, T1 and T2) were employed. All the pre-infection samples resulted *L. infantum*-negative and all post-infection samples tested were *L. infantum*-positive by quantitative real-time PCR of bone marrow aspirates. For bone marrow extraction, dogs were submitted to sedation with medetomidine (Domtor®) and butorphanol (Torbugesic®). At each time point, the presence of *Dirofilaria immitis, Anaplasma Phagocytophylum, Borrelia Burgdorferi,* and *Ehrlichia Canis* antibodies were discharged using the Canine SNAP 4Dx test (IDEXX laboratories, IDEXX Europe B.V., Hoofddorp, The Netherlands). Quantitative polymerase chain reaction (qPCR) of bone marrow was negative results at T0, and positive at T1 and T2 in all the animals (data not show).

For the same four animals, pre-infection samples were selected for T0, those time points in which ferritin and CRP were increased in serum were selected for T1; and the time points corresponding to the development at least two clinical signs that can appear in CanL (such as

anorexia, weight loss, peripheral lymphadenomegaly, cutaneous lesions or fever) were selected for T2 (Table 1). None of the animals presented any abnormalities by external examination at T1.

2.3. Trypsin digestion of serum proteins.

Serum protein extracts (100 µg serum from each dog in every time point), were methanol/chloroform precipitated and resuspended in 30 µl of sample buffer. Proteins were gel concentrated, trypsin digested and the resulting peptides desalted as previously described (Villar et al., 2015). Samples were stored at – 20 ºC until mass spectrometry analysis.

2.4. Serum proteome analysis by SWATH-MS.

The desalted protein digests were resuspended in water containing 2% acetonitrile and 5% formic acid and analysed by reverse phase liquid chromatography coupled online with mass spectrometry (RP-LC-MS/MS) using an ekspertTM nanoLC 415 system coupled to a 6600 TripleTOF® mass spectrometer (AB SCIEX; Framingham, US) through Information-Dependent Acquisition (IDA) followed by SWATH (Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra). Approximately 2 µg of each protein digest of sera from the four dogs in each condition (T0, T1 and T2) were pooled and used for the generation of the reference spectral ion library as part of SWATH-MS analysis. The peptides were concentrated using a 0.1×20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075×250 mm C18 RP column (New Objective, Woburn, MA, USA) operating at 300 nl/min. Peptides were eluted in a 120-min gradient from 5 to 30% solvent A (0,1% formic acid in water) followed by 10-min gradient from 30 to 60% solvent B (0,1% formic acid in acetonitrile) and directly injected into the mass spectrometer for analysis.

For IDA experiments, the mass spectrometer was set to scanning full spectra (390-1400 m/z) using 250ms accumulation time per spectrum, followed by up to 50 MS/MS scans (100-1500 m/z). Candidate ions with a charge state between +2 and +5, and counts per second above a minimum threshold of 100, were isolated for fragmentation. One MS/MS spectra was collected for 100 ms, before adding those precursor ions to the exclusion list for 15 s (mass spectrometer operated by Analyst® TF 1.6, ABSciex®). Dynamic background subtraction was turned off. MS/MS

analyses were recorded in high sensitivity mode with rolling collision energy on and a collision energy spread of 5.

For SWATH quantitative analysis, 5 µg of each independent sample were subjected to the cyclic data independent acquisition (DIA) of mass spectra using the SWATH variable windows calculator (V 1.0, AB SCIEX) and the SWATH acquisition method editor (AB SCIEX), similar to previously established methods (Gillet et al., 2012). A set of 50 overlapping windows was constructed (containing 1 m/z for the window overlap), covering the precursor mass range of 400–1250 m/z. For these experiments, a 50 ms survey scan (390–1400 m/z) was acquired at the beginning of each cycle, and SWATH-MS/MS spectra were collected from 100–1500 m/z for 70 ms at high sensitivity mode, resulting in a cycle time of 3.6 s. Collision energy for each window was determined according to the calculation for a charge +2 ion-centered upon the window with a collision energy spread of 15.

2.5. Library generation/protein identification, data processing and relative quantitation.

To create a spectral library of all the detectable peptides in the samples, the IDA MS raw files were combined and subjected to database searches in unison using ProteinPilot software v. 5.0.1 (AB SCIEX; Framingham, US) with the Paragon algorithm. Spectra identification was performed by searching against a complied database containing all sequences from *Canis lupus familiaris* proteome and *Leishmania* taxonomy (Uniprot databases: 25,491 and 58,760 entries, respectively, in May, 2018) with the following parameters: iodoacetamide cysteine alkylation, trypsin digestion, gel-based ID as special factor, identification focus on biological modification and thorough ID as search effort. The detected protein threshold was set at 0.05. An independent False Discovery Rate (FDR) analysis, using the target- decoy approach provided by Protein PilotTM, was used to assess the quality of identifications. Positive identifications were considered when identified proteins reached a 1% global FDR.

For SWATH processing, up to ten peptides with seven transitions per protein were automatically selected by the SWATH Acquisition MicroApp 2.0 in the PeakView 2.2 software with the following parameters: 15 ppm ion library tolerance, 5 min XIC extraction window, 0.01 Da XIC width, and considering only peptides with at least 99% confidence and excluding those which were shared

or contained modifications. However, to ensure reliable quantitation, only proteins with 3 or more peptides available for quantitation were selected for XIC peak area extraction and exported for analysis in the MarkerView 1.3 software (AB SCIEX). Global normalization was performed according to the Total Area Sums of all detected proteins in the samples. A Student's T-test (Gaussian distribution, paired comparison) was used to perform two-sample comparisons between the averaged area sums of all the transitions derived for each protein across the four replicate runs for each sample under comparison, in order to identify proteins that were significantly differentially represented between samples (T0 to T1, T0 to T1 and T1 to T2). Fold changes (FC) have been calculated as follow FC= Group 2/Group 1, being T1/T0, T2/T0, and T2/T1 for their respective comparisons. The mass spectrometry proteomics data have been deposited at the PeptideAtlas repository (http://www.peptideatlas.org/) with the dataset identifier PASS01352.

2.6. Bioinformatics and GO pathways

Proteomics data were used for Gene Ontology (GO) analysis. Canine genes encoding proteins differentially expressed were converted to their human orthologs using the Ensembl orthologs database and its BioMart tool for data mining (www.ensembl.org). Obtained genes were used to determine the GO terms over-represented in three conditions (T0, T1, and T2), by the utilization of through Protein Analysis Through Evolutionary Relationships (PANTHER) classification tool.

Venn diagrams of proteins differentially expressed were drawn through the online software "Calculate and draw custom Venn diagrams" (http://bioinformatics.psb.ugent.be/webtools/Venn/).

2.7. Haptoglobin verification

For haptoglobin (Hp) verification, serum samples from client-owned dogs positive to CanL by serology presented at private veterinary clinics of Murcia Region for routine check-ups were assessed for inclusion. Dogs were classified as symptomatic and asymptomatic according to external examination performed by licensed veterinarians. The dogs were excluded of the study if they presented other diagnosed pathologies. Samples were collected on the morning after an

overnight fasting by cephalic venepuncture. Blood was clotted at room temperature and centrifugation (3700g for 10 min, 20ºC) was performed for serum separation.

Serum haptoglobin concentration was measured by a commercially available colorimetric method (kit haptoglobin Tridelta phase range, Tridelta Development) that was previously validated for its use in dog (Martinez-Subiela & Cerón, 2005). Haptoglobin was expressed in g/L. The results for haptoglobin were evaluated for normality of distribution using D'Agostino and Pearson omnibus normality test statistics. Since the date was non-normally distributed, Mann–Whitney U test was used to compare concentrations. Analysis was performed using statistical software (GraphPad), and values of $P < 0.05$ were considered significant.

3. Results

3.1. Quantitative proteomics analysis revealed changes in serum proteome during the course of CanL infection

Proteomics analysis allowed the identification of 169 proteins from *Canis Lupus familiaris* proteome with FDR ≤ 1% from the eighteen non-depleted canine serum samples (Suppl table 1). A total of 44 proteins were differentially represented between the different time periods: 1 protein between T0 and T1, 13 when compared T0 and T2, and 30 proteins between T1 and T2 (Table 2). No proteins were identified by comparing our results with Leishmania taxonomy database.

Complement C8 alpha chain (C8A) was the only protein that changed in abundance between T0 and T1, with a 2.24-fold decrease. At T2, sphingomyelin phosphodiesterase acid like 3A (SMPDL3A), leucine rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain (ITIH2), hyaluronan binding protein 2 (HABP2), serpin family A member 3 and member 4 (SERPINA 3, and SERPINA 4, respectively), complement C9 (C9), and sulfhydryl oxidase (QSOX1) were over-represented when compared to pre-infection levels. When the subclinical (T1) and clinical stages (T2) were compared, adiponectin (ADIPOQ) and transferrin (TF) were the only proteins under-represented at T2 when compared to T1, while C8A, SMPDL3A, tranketolase (TKT), LRG1, apolipoprotein A-IV (APOA4), lecithin-cholesterol acyltransferase (LCAT), fibrinogen

gamma chain (FGG), HABP2, lymphocyte cytosolic protein 1 (LCP1), haptoglobin (HP), hepatocyte growth factor activator (HGFAC), and SERPIN A3 were over-represented at T2.

3.2. Identification of biological processes involved in CanL by proteomics and bioinformatics analyses

The biological processes in which the proteins differentially expressed between the different stages of CanL infection are shown as Venn diagrams in Figure 1a. Complement C8 alpha chain was identified as the only common protein significantly modulated when comparing T0 vs T1 and T1 vs T2. Nine common proteins, identified as sphingomyelin phosphodiesterase acid like 3A, hyaluronan binding protein 2, leucine rich alpha-2-glycoprotein 1, serpin family A member 3, and still uncharacterized proteins L7N0P9_CANLF, F1PF90_CANLF, F1PQU0_CANLF, F1PG16_CANLF, and J9NSQ1_CANLF, were found significantly modulated when T0 vs T2 and T1 vs T2 were compared. In addition, four proteins were differently represented only in T0vsT2, while 20 proteins showed differences only between T1 and T2.

These 44 proteins differentially expressed between the different time points were used for a qualitative analysis in terms of functional clusters, according to the PANTHER classification system (http://www.pantherdb.org) (Figures 1b-d). The identified differentially modulated proteins between the different time points were distributed into 4 different molecular functions namely catalytic activity (47.4%), binding (31.6%), molecular function regulator (15.8%), and transporter activity (5.3%). These proteins are involved in 10 different biological processes, being response to stimulus, metabolic process, and biological regulation the most represented ones with 34.2, 18.4, and 13.2% of proteins, respectively. Regarding biological pathways, the differentially modulated proteins participated in blood coagulation, gonadotropin-releasing hormone receptor pathway, pentose phosphate pathway, and plasminogen activating cascade (25% of proteins for each one).

3.3. Haptoglobin verification

A total of 60 dogs of different breeds were included for haptoglobin verification. Thirty-three dogs (47% males, 6.8 ± 2.95 years old) were classified as symptomatic, while twenty-seven (37% males, 5.9 ± 2.6 years old) were considered as asymptomatic. No statistical significant difference was detected in age between the two groups (p=0.301).

Statistically higher serum concentrations of Hp were observed in dogs with clinical signs of CanL when compared to asymptomatic positive to *Leishmania* dogs (4.62 [3.08 – 5.28] versus [2.36 [1.15 – 5.17] g/L, respectively) (p= 0.013).

4. Discussion

In the present study, a proteomics study in serum of healthy (pre-infection), subclinical (asymptomatic), and clinical symptomatic stages of canine leishmaniosis was performed using TMT. The use of an experimental infection model allowed the study and monitoring the changes in the protein expression patters over time in the same animal. The subclinical stage was selected according to previously published criteria in which dogs do not have external clinical signs of infection but an increase in acute phase proteins, this being considered as a phase leading to the clinical stage that can differ in length depending of the individual (Paltrinieri et al., 2010).

In dog sera, the complement C8 alpha chain (C8A) was the only protein that was significantly decreased at pre-symptomatic phase (T1) in comparison to pre-infection (T0) values. Complement C8 is a constituent of the membrane attack complex being inserted into the target membrane forming pores (Hadders, Beringer, & Gros, 2007). The activation of complement has proven to be partially effective against *Leishmania* promastigotes, being able to kill most of the parasites (Solbach & Laskay, 1999). Complement activation has been correlated with absence of parasites in the skin macrophages of following cutaneous *Leishmania* inoculation (de Amorim et al., 2011). Thus, the decrease on C8A at the pre-symptomatic phase could be compatible with a less effective response of the dogs and the subsequent onset of the disease.

When abundances of proteins were compared between subclinical (T1) and symptomatic (T2) phases, adiponectin (ADIPOQ) and transferrin (TF) were the only proteins that significantly decreased after the development of clinical signs of CanL. Adiponectin is produced in the liver

and reduces plasma glucose concentrations besides promoting fatty acid oxidation. In addition, adiponectin is related to inflammation and immune response (Berg & Scherer, 2005; Yamauchi et al., 2002), being down-regulated in several inflammatory conditions such as obesity and metabolic syndrome (Asta Tvarijonaviciute et al., 2012), acute pancreatitis(Paek et al., 2014), and diabetes mellitus (Kim, Kim, Kang, & Yang, 2015). In CanL, adiponectin has been shown to be down-regulated in sera of dogs with clinical signs, when compared to healthy dogs (A. Tvarijonaviciute, Ceron, Martinez-Subiela, & García-Martinez, 2012), which is in concordance with our results. However, to the authors' best knowledge, the decrease of adiponectin in symptomatic dogs in comparison with the pre-symptomatic phase was not previously reported. On the other hand, transferrin is responsible for the iron transport into the circulatory torrent and acts as a negative acute phase protein in dogs (Cerón, Eckersall, & Martínez-Subiela, 2005). Similarly to our results, decreased serum transferrin levels have been reported in symptomatic dogs with CanL when compared to healthy animals due to the caused inflammation (Burillo, Perez, Liesa, & Fabian, 1994; Silvestrini et al., 2014) being increased and restored after an effective anti-leishmania treatment (Martínez-Subiela et al., 2017). However, there are no reports of serum transferrin levels among the different stages of CanL. Overall, the data obtained in this manuscript with increases in ferritin and decreases in transferrin indicate that there is an alteration in iron metabolism in CanL. Although it was not measured in this study, the increase in ferritin and decrease in transferrin would indicate an increase of storage of iron and decrease in its transportation to blood respectively, that would lead to a decrease in serum iron, as it has previously described in dogs with clinical leishmaniosis (Silvestrini et al., 2014).

The abundance of a total of 28 proteins showed to be increased at T2 when compared to T1, being from 1.48 to 3.21-fold higher. Among them, C8A and several uncharacterized proteins (compatible with immunoglobulins according to their BLAST sequence) were the most up-regulated. This increase of C8A level could be related to the activation of complement cascade, similarly to that described in the case of other inflammatory diseases such as canine babesiosis (Kuleš et al., 2014) or parvovirosis (Franco-Martínez et al., 2018). Thus, the increase in C8A could point out the activation of complement cascade in response to the diffusion of Leishmania through the organism. It is interesting to note the dynamics of C8A in the experimental infection, with a lower expression level at the initial stages of the sickness when the animal is at the

subclinical stage and an increased level when the clinical signs appear. The observed progressive increase in the serum level of several immunoglobulins during the development of CanL was expected since hyperglobulinemia was detected by serum biochemistry (Table 1) and because it has been extensively studied in CanL, being serological assays based on the detection of specific anti-*Leishmania* antibodies in serum considered as one of the current diagnostic techniques (Laia Solano-Gallego et al., 2014). According to their BLAST sequence, different types of immunoglobulins were found to increase in abundance during the onset of the disease, which may point out different mechanisms such as the enhancement of Th2 response against the infection -which has been proven to be an ineffective response against leishmaniosis leading to the onset of the disease-, and also the development of a non-specific antibody production, as described previously (Silva-Barrios et al., 2016). In the clinical stage of the disease (T2), all the proteins that were significantly modulated in comparison with pre-infection (T0) measurements were up-regulated, with increasing rates ranging from 1.45 up to 3.48-fold higher. Of these 13 proteins, two uncharacterized proteins (L7N0P9_CANLF and F1PF90_CANLF) and sphingomyelin phosphodiesterase acid like 3A (SMPDL3A) were the most up-regulated. BLAST analyses and sequence comparison with proteins of know function suggested that the two uncharacterized proteins corresponded to immunoglobulins (immunoglobulin kappa light chain variable region [*Canis lupus familiaris*] [89.34% identity; 96% query cover] and immunoglobulin iota chain-like [*Canis lupus familiaris*] [95.65% identity; 99% query cover], respectively). SMPDL3A is a nucleotide phosphodiesterase for nucleoside triphosphates such as ATP, and lesser activity with nucleoside diphosphates. This protein is detected in macrophages, and is known to be up-regulated in macrophages in response to cholesterol accumulation and by cAMP (Traini et al., 2014). Since extracellular nucleotides may activate pro-inflammatory responses in immune cells, SMPDL3A has been proposed as a potentially novel anti-inflammatory protein by decreasing the concentration of these pro-inflammatory nucleotides (Traini et al., 2014). According to Panther system classification, the differentially modulated proteins that were found among the subclinical and clinical stages of CanL are mainly related to blood coagulation, gonadotropin-releasing hormone receptor pathway, pentose phosphate, and plasminogen activating cascade pathways, posing different molecular functions such as catalytic activity, binding, molecular

function regulation, and transporter activity. Therefore, these systems and functions are affected when the disease is symptomatic.

Finally, one of the identified proteins by proteomic studies was verified as a possible marker for the follow-up of canine leishmaniosis. Haptoglobin was selected because it could be easily measured by validated methods for its use in dogs; and because it has been previously proposed as biomarker for CanL diagnosis (Cantos-Barreda et al., 2018) or response to treatment, decreasing after an effective *anti-leishmania* treatment (Martinez-Subiela et al., 2011). However, to the best of the author's knowledge, this is the first report in which Hp is observed to change by a high-throughput proteomic approach during the development of CanL in experimentally infected dogs; followed by its verification in dog naturally infected by *Leishmania* at symptomatic and asymptomatic stages. Our results showed that Hp concentrations were higher in symptomatic dogs in both experiments, which was in agreement with previous literature (Cantos-Barreda et al., 2018), proposing serum Hp as a feasible biomarker of diagnosis and severity of CanL. Further studies would be desirable to verified other differentially represented proteins as possible biomarkers for CanL.

The results obtained in the present study should be interpreted with caution due to the low sample size and because the changes observed after the experimental infection could differ from those dogs naturally infected and among the different clinical stage. Thus, the corroboration of our results with in a larger amount of dogs, and also compared with those obtained from naturally infected dogs at the different clinical stages of CanL according to the established guidelines such as the Leishvet would provide with further insight in the early detection or predicting the outcome in canine leishmaniosis. Finally, the proteomic approach employed in the present study could be of utility to the further knowledge of other diseases such as canine ehrlichiosis, babesiosis or filariosis, and have already applied for other important canine diseases such as parvoviral enteritis. The comparative analysis of the changes in serum proteome due to these diseases and in animals presenting co-infections could be of great value in the understanding of the pathological mechanisms of the diseases and for the search for new biomarkers for its detection, monitoring or prognosis.

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Conflict of interest

The authors report no other conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1. Main clinicopathological findings in dogs with clinical CanL and clinical stages based on LeishVet guidelines at the moment of T2 (Laia Solano-Gallego et al., 2011).

		T1				T2						
	Gender / age in years / weight in kg	Weight	Days from T0	CRP	Ferritine	Weight	Days from T0	CRP	Ferritine	External examination	Laboratory abnormalities	Clinical stage (Leishvet)
1	Male/ 1 /17.5	17.2	168	34.2	421.4	16.1	211	97.7	901.5	Lymphadenomegaly Cutaneous lesions (exfoliative and ulcerative dermatitis) Ocular lesions (blepharoconjunctivitis)	Hypoalbuminemia Hyperglobulinemia Increased CRP and ferritine	II a
2	Male/1 /18.5	18.3	168	6.8	263.1	17.1	211	38.7	748	Lymphadenomegaly Cutaneous lesions (exfoliative and ulcerative dermatitis) Ocular lesions (blepharoconjunctivitis)	Hypoalbuminemia Hyperglobulinemia Increased CRP and ferritine	II a
3	Female /1 /15.8	15.7	168	38.5	664.2	16	269	30.4	1273.5	Lymphadenomegaly	Hyperglobulinemia	II a

										Ocular lesions (blepharitis)	Increased CRP and ferritine	
4	Female/ 6/13.3	13.4	168	28.5	376	12.5	241	7.6	240.7	Lymphadenomegaly	Normocytic hypochromic anemia Hypoalbuminemia Hyperglobulinemia Increased CRP, ferritine, ALP, AST and ALT	II a

Interlab-UMU biochemical laboratory reference values: CRP: <12 µg/mL; Ferritin: <190 µg/L.

Table 2. Identification by SWATH-MS of differentially expressed seric proteins in dogs before (T0) and after (T1 and T2) experimental infection with *L. infantum*.

Accession number	Protein identity	T1/T0	T2/T0	T2/T1
E2R109	Complement C8 alpha chain	0,38		3,49
L7N0P9	Uncharacterized protein †		3,48	2,75
J9NYQ3	Sphingomyelin phosphodiesterase acid like 3A		2,98	2,57
F1PF90	Uncharacterized protein †		2,30	3,49
J9NSS7	Leucine rich alpha-2-glycoprotein 1		1,96	2,20
F1PQU0	Uncharacterized protein		1,88	2,64
F1PG16	Uncharacterized protein		1,81	1,86
J9NSQ1	Uncharacterized protein †		1,78	3,16
F1PG39	Inter-alpha-trypsin inhibitor heavy chain 2		1,68	
F1Q1K9	Hyaluronan binding protein 2		1,67	1,77
F1PH87	Serpin family A member 3		1,48	1,47
F1PHN8	Serpin family A member 4		1,47	
E2RFV9	Complement C9		1,45	
F1PLT8	Sulfhydryl oxidase		1,45	
J9P0D4	Uncharacterized protein †			3,25
F1PKN5	Uncharacterized protein †			2,59
F1PE28	Transketolase			2,46
F1PDJ7	Uncharacterized protein			2,42
APOA4	Apolipoprotein A-IV			2,18
J9NWG4	Uncharacterized protein			1,98
L7N0E7	Uncharacterized protein †			1,92
J9PAW5	Uncharacterized protein †			1,89
F1PA63	Lecithin-cholesterol acyltransferase			1,88
E2QW61	Uncharacterized protein			1,85
J9P6L3	Uncharacterized protein †			1,85
J9P1F6	Uncharacterized protein †			1,83
F1P8G0	Fibrinogen gamma chain			1,83
E2QWN7	Lymphocyte cytosolic protein 1			1,72
G1K2D9	Haptoglobin			1,63
F1Q184	Uncharacterized protein †			1,63
J9P050	Uncharacterized protein †			1,58
F1PAF3	Hepatocyte growth factor activator			1,49

A0A0B4J198	Adiponectin, C1Q and collagen domain containing	0,76
J9P430	Transferrin	0,71

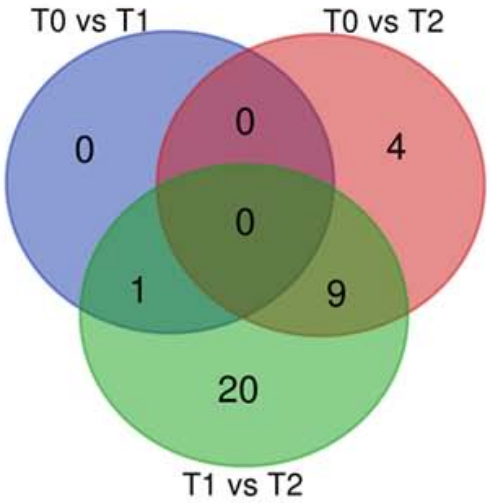
* Accession number from Uniprot protein database for *Canis lupus familiaris* (www.uniprot.org)

[+] Proteins compatible with immunoglobulins based on BLAST sequences.

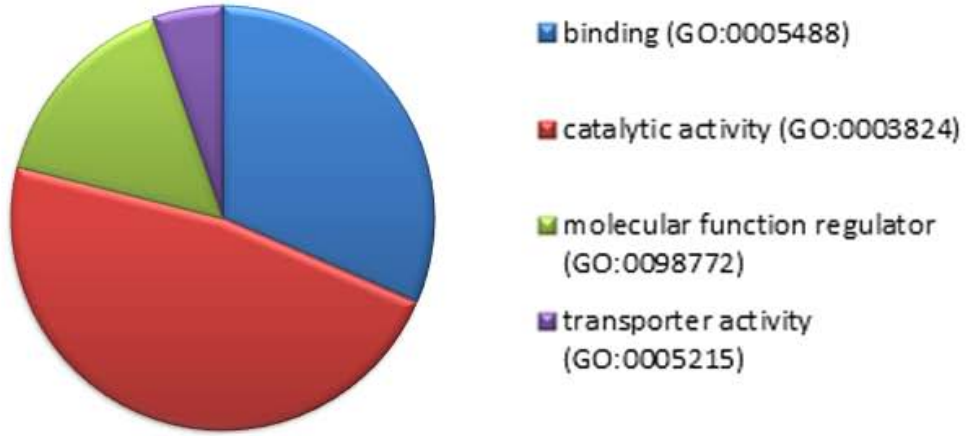
Figure legends

Figure 1. a) Venn Diagrams showing the unique and common differentially represented proteins at the different times performed. One common protein was identified as significantly modulated when comparing T0vsT1 and T1vsT2. 9 common proteins were identified as significantly deregulated in symptomatic animals when comparing T0vsT2 and T1vsT2. Additionally, T0vsT2 and T1vsT2 showed 4 and 20 unique proteins differentially expressed, respectively. b-d) Pie chart showing the molecular function, biological process, and pathways as a percentage of the 44 distinct identified proteins based on the PANTHER classification system.

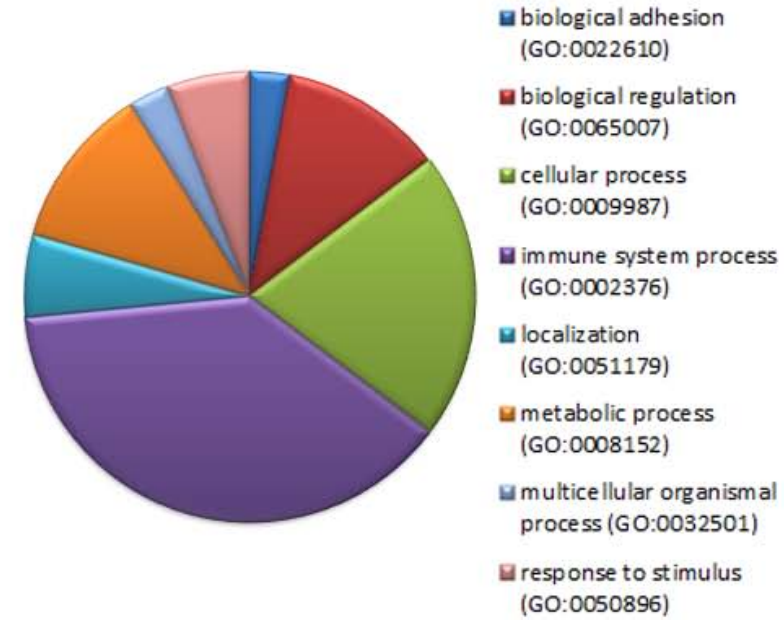
a)


T0 vs T1
T0 vs T2
T1 vs T2
0
0
4
0
1
9
20

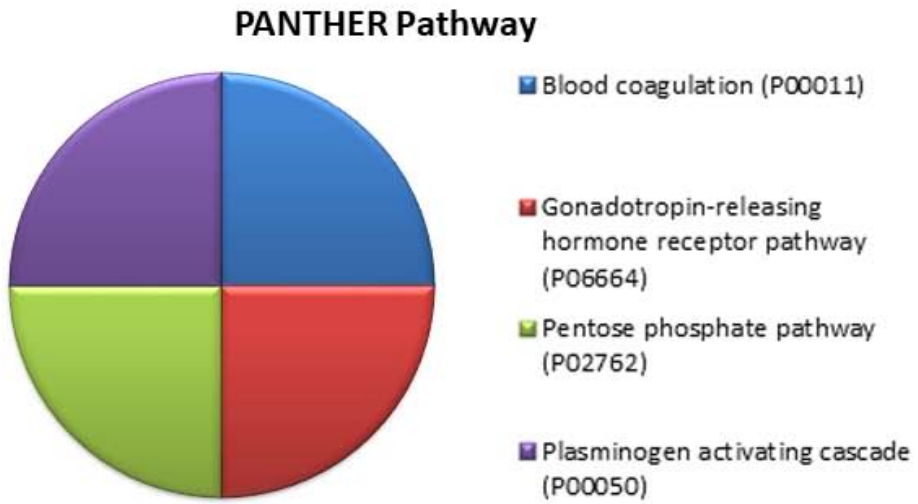
b)


Panther GO-Slim Molecular Function
binding (GO:0005488)
catalytic activity (GO:0003824)
molecular function regulator (GO:0098772)
transporter activity (GO:0005215)

c)


Panther GO-Slim Biological Process
biological adhesion (GO:0022610)
biological regulation (GO:0065007)
cellular process (GO:0009987)
immune system process (GO:0002376)
localization (GO:0051179)
metabolic process (GO:0008152)
multicellular organismal process (GO:0032501)
response to stimulus (GO:0050896)

d)


PANTHER Pathway
Blood coagulation (P00011)
Gonadotropin-releasing hormone receptor pathway (P06664)
Pentose phosphate pathway (P02762)
Plasminogen activating cascade (P00050)